Proposed title for the Ph. D. work: Interactive toxicity of Arsenic and Fluoride on Zebrafish (*Danio rerio*) and Human Peripheral Blood Mononuclear Cells

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Reg. no. - VB-2261 of 2016-17

Date of registration: 28.11.2016

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Keywords: Fluoride, Arsenic, Zebrafish, Co-exposure, oxidative stress

Introduction

Contaminants occur in different combinations in natural environment. Environmental policies have identified 'mixture effects' as a major issue in risk assessment (Altenburger R. *et al*. 2012). The interaction of contaminants in a complex environment is known to influence their bioavailability, uptake, tissue specific bioaccumulation and resultant biological responses. Furthermore, these contaminants may act simultaneously as carcinogen, mutagen and reproductive (CMR) toxicants and cause other severe health impacts, which have raised considerable regulatory concerns (Dallas LJ. *et al*. 2013). While extensive work has been conducted with respect to the genotoxic, mutagenic and reproductive toxicant potential of arsenicals for both humans and aquatic species (Liu J. *et al*., 2007, Boyle D. *et al*., 2008) , there is a lack of information with respect to the toxicity of F either alone or in combination with As, bearing in mind that both of them may occur concurrently in the environment. Thus populations are chronically exposed and chronic poisoning resulting from both As and F is becoming endemic in India. Of particular note, the alluvial flood plains of the river Ganges and Brahmaputra provide groundwater reported to have As and F beyond permissible limits (Kumar M. *et al*., 2016, Dutta J. 2013) . The impact of such co-occurrence has not been investigated in India, although both As or F have been reported in ground water. In addition, the presence of As and F in irrigation water affect crops and pose risks in the food chain that are detrimental for human health. The presence of As in rice has been widely reported from different parts of the world (Mondal D. & Polya DA., 2008) although the effect of co-occurrence of As and F on plants is much less studied, with a few reports on soy bean, red clover and broad bean grown under pot experiments on spiked soils either individually or simultaneously (Bustingorri C. *et al*., 2015, Sadee BA *et al*., 2016) . Furthermore, epigenetic modification of key genes involved in DNA damage/ repair and hormonal synthesis could pose enhanced risk to As exposed populations as shown previously by our group (Bandyopadhyay A. *et al*. 2016) . In the above context, to calculate the total mutagenic/ carcinogenic risk, several researchers have simply added the risk contributions (potency × dose) from co-existing environmental carcinogens. In some cases, this additive model is justified, but their combinational action demands rigorous research. Synergistic effects have been found when combining the exposure to smoking and arsenic, and it can be expected that other contaminants may interact similarly. It is imperative that we probe this hypothesis to advance the science beyond the current state-of-the-art knowledge base. This is particularly pertinent since emerging new technologies, including nanotechnologies, have raised new questions relating to 'interactive toxicology' under chronic, low level exposure conditions and how best to potentially remove the contaminants from the water supply to protect our health. Once resolved this could also help to develop strategies to set values for regulatory compliance, effective and concurrent remediation and to develop better management plans.

Arsenic is a metalloid that occurs naturally in the earth's crust and is a component of more than 200 minerals. Its inorganic forms are mainly arsenite and arsenate compounds and are

recognized worldwide as toxic to human health. Arsenic is classified as a Group I human carcinogen (IARC Monograph, 1980) and can induce chromosome aberrations (CAs), sister chromatid exchanges (SCEs) in human *in vivo* as well as in various cell types *in vitro*. Number of studies has reported that arsenic is neuro, hepato as well as nephrotoxic both in mammals as well as in fish. Though ground water pollution is the major route for arsenic pollution, evidences are accumulating that significant concentrations of arsenic is deposited in rice, the staple food in India and many other south Asian countries, compounding the threat of spread of arsenic pollution in those areas where ground water is not the main source.

Fluoride (F) ingestion through drinking water or other sources has become a serious endemic health problem in 29 nations including India, China and Mexico affecting 200 millions of people all over the world. Intake of excessive amounts of F, mostly from water, and less often from food and air lead to fluorosis. Several reports are available on the toxic effects of F on the various organs including brain reproductive tissues, liver, kidney, pancreas and spleen.

In recent time, interaction of arsenic and fluoride received significant attention because concurrent exposure of these toxicants is reported in various countries like India, China, Mexico and Latin American countries. Although the toxic effects of arsenic and fluoride when administered alone have been widely studied and are relatively well-known, the biological effects and possible interactions when the exposure is simultaneous have only been scarcely studied, and the results of these studies are controversial and the mechanism of action is not still understood. Antagonistic, synergistic, and independent effects have all been reported.Both arsenic and fluoride act through the generation of reactive oxygen species where Nrf2 acts as the master regulator of antioxidant and stress related genes through its antioxidant responsive elements ARE. The modulation of the expression of these genes in different organs after individual and combined exposure to arsenic and fluoride can give insight into the cellular response to individual and combined exposure to these two major toxicants which have significant global concern.

Relevance of study

Arsenic is the 33rd element in the periodic table. It exists in the metallic state in nature in three allotropic forms and in several ionic forms. Arsenic occurs in many minerals, these are mostly ores containing sulfides, along with copper, nickel, lead, cobalt, or other metals and also as a pure elemental crystal. Arsenic in drinking water has been reported from six districts of West Bengal (GuhaMazumder *et al*.,1988). Arsenic is ubiquitous in nature and found in soil, water, rocks, air and food. There are trace amount of arsenic in all living matter.Sea food contains organic arsenicals (arsenobetaine), arseno sugars and arseno lipids. Marine products usually contain organic arsenic in higher concentrations which is nontoxic and is excreted unchanged (Navas–Acein *et al*., 2008).

Exposure to arsenic results into many adverse health effects, including cardiovascular system, hepatic and renal disease in addition to cancer in kidney, liver, lungs, urinary bladder and skin(Kitchin K T, 2001). One of the hallmarks of chronic toxicity in humans from oral

exposure to arsenic is skin lesions, which are characterized by hyper pigmentation, hyperkeratosis, and hypopigmentation (Yeh *et al.*, 1968). In Taiwan, black foot disease , a vasoocclusive disease which leads to gangrene of the extremities, is also observed in individuals chronically exposed to arsenic in their drinking water (Tseng, 1977).

Fluorine (F) often found in the earth´s crust (13th most abundant element), is highly electronegative and a member of the halogen family (O' Donnell, 1973). Fluorine compound, are naturally present in soil, water and food, is an essential trace element for human being and animals and elemental fluorine is a pale yellow green, irritating gas with a sharp odor and atomic mass of 18.998. F occurs naturally in the environment and is widely distributed in fresh and marine water. It is continuously increasing due to anthropogenic activities like rapid industrialization, use of fluoridated pesticides and municipal water discharges in rivers. Fluoride is a natural component of soil and water, in which it is mainly bound in complexes and not readily leached. The major source of free fluoride ion in soil is the weathering and dissolution of local igneous and metamorphic bed rocks.

Fluoride shows detrimental effect on skeletal systems, teeth, and also the brain and spinal cord (Shashi *et al*., 1992).General symptoms of acute fluoride poising include nausea, salivation, vomiting, diarrhea and abdominal pain. Chronic toxicity occurs after the long term ingestion of small amount of fluoride. Symptoms include emaciation, stiffness of joints and fluorosis of teeth and bones. Fluoride was found to be an equivocal carcinogen by the National Cancer Institute Toxicological Program (Maurer *et al*., 1990). He *et al*. (2006) reported that excess fluoride can induce oxidative stress and DNA damage and lead to apoptosis and cell cycle change in rat oral mucosal cells and hepatocytes.. Fluoride is reported to be hepatotoxic in mouse (Chattopadhyay *et al*.,2011). Mukhopadhyay *et al*.,(2014) showed that sodium fluoride exposure to zebrafish induces oxidative stress and alters gene expression in liver. Mukhopadhyay *et al*.(2015) again reported that exposure to sodium fluoride generates oxidative stress and alters transcription of genes for xenobiotic metabolizing enzymes in adult zebrafish liver and affects zebrafish behavior and alters mRNA expression of biomarkers genes in the brain.

Epidemiologic studies have shown that co-exposure to fluoride and arsenic increased the risk of reduced IQ scores in children of Shanyin county, Shanxi province of China as well as in Mexico (Wang *et al*., 2007; Rocha-Amador *et.al* 2007). Several studies conducted in arid and semi-arid regions of Latin America show that the co-occurrences of arsenic and fluoride in drinking water. These types of contamination are particularly pronounced in arid and semi-arid regions, where high arsenic concentrations often show a direct relationship with high F concentrations (Alarcón-Herrera *et al*., 2012). Jiang *et al*. (2014) reported that compared with controls, glutamate levels decreased in the hippocampus and cortex of rats exposed to fluoride and combined fluoride and arsenic, and in cortex of arsenic-exposed rats. Study showed that residents of Chihuahua of Mexico, who were exposed to high arsenic concentrations in drinking water, were also exposed to high levels of fluoride, raising questions about possible contribution of fluoride exposure to the adverse effects that have so far been attributed only to arsenic

exposure. It was concluded that investigation of possible interactions between arsenic and fluoride exposures and its related health risks deserves immediate attention (González-Horta *et al*., 2015).

Flora and his group performed some studies in experimental models with the aim of evaluating the effect of arsenic and fluoride, individually or together, on oxidative stress and cell injury (Mittal *et al*., 2006). It was concluded that the simultaneous administration of arsenic and fluoride is less toxic, or may even afford an antagonistic effect (Flora *et al*.,2009) compared to when these toxicants are administered individually. This discrepancy could be explained by the differences in the biological systems used, or by the dosages or proportions of the combined doses of both pollutants. Report is also there that simultaneous exposure to both xenobiotics increased the induction of genotoxic effects (Tiwari, 2010). Tea garden belt of Sonitpur district of Assam was found to have high level of arsenic, fluoride and other heavy metals (Dutta, 2013).

Thus arsenic and fluoride aredetrimentalnot only to the place where these are actually present but also in less contaminated areas. Contamination isalso conveyed to the noncontaminated place by staple food such as rice,wheat. So it is necessary to evaluate the combined toxic effect of arsenic and fluoride and their mechanism of toxicity.

Objectives:

1. To determine the LC_{50} of arsenic and fluoride on zebrafish (*Danio rerio*) and human peripheral blood mononuclear cells (HPBMCs).
2. The individual and combined cytotoxic effect of arsenic and fluoride in liver, gill and brain through histopathology and biochemical assay.
3. Expression pattern of antioxidant biomarker genes and genes of Phase I and Phase II xenobiotic metabolizing enzymes (XMEs) at transcriptional and translational level.
4. Expression pattern of Nrf2/Keap1 mRNA and protein to understand their regulation on the related antioxidant and XME genes.
5. To study individual and combined exposure of arsenic and fluoride induced cell death in zebrafish hepatocytes.
6. Study of *in vitro* toxicity on HPBMCs by cell viability assay, cell cycle analysis and chromosome aberration studies.

Toxicity studies of individual and combined exposure of arsenic and fluoride in zebrafish (*Danio rerio*):

Toxicity test of chemicals on animals has been used for a long time to detect the potential hazards posed by chemicals to man. Fishes have been extensively used as test organisms for acute toxicity bioassays due to several advantages. According to U.S. Environmental Protection Agency, they are easily maintained under laboratory conditions, and they are sensitive to a variety of pollutants as well as generally available throughout the year from both commercial and

natural sources. Among freshwater animals, zebrafish, Danio rerio are widely used for vertebrate embryo toxicity bioassays. Evidence has long suggested that zebrafish is a good model to predict toxicity of various compounds that are toxic to humans. It is now widely used in many research areas due to several advantages like easy availability, low maintenance cost and breeding in laboratory condition as well as similarity with human genome.In vivo studies will be done on zebrafish treated to sub lethal concentration of arsenic and fluoride subsequent to determination of LC50.

Methodology

Exposure of zebrafishto arsenic trioxide (As_2O_3) and sodium fluoride (NaF)

Adult female zebrafish (average weight of 0.7±0.01 g and length of 3.6±0.05 cm) will be procured from local supplier and acclimatized to soft water in the laboratory condition for two weeks prior to the experiments. Fish will be kept in aquaria with uniform water temperature (26 ± 1^0C), pH (7.0-7.2) and a photoperiod (14:10 h light : dark cycle) and fed daily with commercial food. Fish (10 fish per group in triplicate sets) will be randomly selected for experiments and exposed to either As_2O_3 orNaF or to both for different time periods with different concentrations of arsenic and fluoride lying within the reported range of arsenic and fluoride levels in different water bodies across the world. Proper control groups will be used for comparisons with corresponding treatment groups. Liver, gills and brain tissues will be used for histopathology and the samples from each set (10 fish) will be pooled for biochemical, mRNA and protein expression studies. Data of three such sets will be used for statistical analysis (n=3).

Detecting toxic effects of individual and combined exposure of arsenic and fluoride:

Determine the lethal concentration of arsenic and fluoride

The potential toxicity of chemical substances often is presented as their $LC_{50.}$ LC_{50} is the concentration of a substance that is lethal to 50% of the organisms in a toxicity test.LC_{50} can be determined for any exposure time, but the most common exposure period is 9 hours.To address this issue,10 fish will be maintained in a glass tubs filled with 1L of testing water and exposed to different concentration of As^{+3}{the procedure is same for fluoride LC_{50} determination}.The mortality will be recorded ever 24 h till completion of 96h.The concentration that will kill 50%of the experimental animals(Zebrafish) will be recorded as $LC_{50.}$

Histological analysis to detect alteration in tissue ultrastructure

Histological study is an integrated parameter, providing a more complete evaluation of the organism’s health, effectively mirroring the effects of exposure to a particular toxicant. To address this issue, at the end of acute exposure time (96 h) fish of all groups will be sacrificed. Tissues from control and treated fish will be dissected out, fixed in Bouin's Fixative. After 24 h

of fixation the tissues will be subjected to series of ethanol concentration for dehydration followed by embedding in a paraffin block. it will be cut into sections of 6 µm onto a glass slide and stained with hematoxylin-eosin for histological assessment according to Bancroft *et al.* (1996). Observation of the prepared slides under light microscope may reveal some changes encountered due to individual and combined toxicity of arsenic and fluoride in fish tissue.

Reactive Oxygen Species (ROS) level determination

ROS levels in the fish liver, kidney and brain will be determined by the oxidation of 2', 7'-dichlorofluorescin diacetate (DCF-DA) according to the procedure described by Contreras *et al*. (2005). Samples of pooled tissues (0.1-1 g wet wt) were incubated in100 ml of 10 µM DCF-DA in methanol for 30 min in a water bath at 37^0C. After incubation, fluorescence of DCF was determined in spectrofluorometer (SpectraMax, Molecular Devices, USA) at an excitation wavelength of 488 nm and emission wavelength of 525 nm. Fluorescence values were obtained using a standard curve prepared with 0-500 nM DCF.

Protein Estimation

Tissues will be homogenized and centrifuged at 10,000g, 20 min at 4 °C. After centrifugation, the clear supernatants will be pipetted out and kept in a fresh tube. These will be used as protein samples and their concentrations will be determined following the method of Lowry *et al*. (1951). Bovine serum albumin (BSA) will be used as the standard.

Measurement of Glutathione (GSH) Level

The level of GSH in liver, kidney and brain will be measured by the method of Akerboom and Sies (1981). Briefly, freshly collected tissues will be homogenized and lysed by alternate freezing (10 min) and thawing (10 min) three times at 0°C and 28°C and centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant will be deproteinized using 100 µl ice-cold 10% 5-sulphosalicylic acid with intermittent shaking and centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant will be immediately used for GSH estimation. 50 µl sample suspension will be added to a reaction mixture containing 1 ml buffer (0.1 M EDTA phosphate buffer, pH 7.0); 50 µl NADPH (4 mg/ml); 20 µl DTNB (1.5 mg/ml); 20 µl GSH reductase (6 units/ml), mixed thoroughly and the optical density was measured continuously for 5 min at 412 nm in a UV-visible spectrophotometer (Beckman Coulter) against a sample blank.

Thiobarbituric Acid Reactive Substances (TBARS) level Estimation

The level of lipid peroxidation as estimated by TBARS, will be determined according to the method of Buege and Aust (1978). Briefly, 1ml of microsomal sample was mixed with 2 ml of TBA-TCA-HCl mixture thoroughly and heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate will be removed by centrifugation at 1000g for 10 min. The absorbance of the supernatant will be determined at 535 nm. The MDA concentration of the

sample will be calculated using an extinction coefficient of $1.56x10^5$/M/cm and expressed in terms of nM MDA/mg protein.

Enzyme Extraction & Biochemical Assays

For enzymatic activities, tissues will be homogenized in phosphate buffer. Homogenates were centrifuged at 10,000g for 20 min at 4 °C. The supernatant of each sample will be employed for biochemical measurements.

Catalase (CAT) activity will be assayed following the procedure of Aebi (1984). The sample (20 μL) will be added to 980 μL of an assay buffer containing 50mM Tris-HCl (pH 8.0), 9mM H_2O_2, and 0.25mM EDTA to constitute the assay volume of 1 mL. The decrease in ΔOD/min of that assay mixture will be recorded at 240 nm for 1min.The results will be expressed as U/mg protein.

Glutathione-S-Transferase (GST) activity will be determined in fish liver cytosolic fractions as described by Habig *et al*. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in presence of excess GSH (5 mM). The rate of CDNB conjugation will be estimated by direct spectrophotometry at 340 nm for 3 min. The result will be expressed as nM GS-CDNB formed/min/mg protein.

Superoxide dismutase (SOD) activity will be assayed following the procedure of Ewing and Janero (1995) using nitrobluetetrazolium (NBT). The specific activity will be expressed in the units of SOD per mg of protein. One activity unit will be defined as the amount of enzyme required for the inhibition of initial rate of NBT reduction by 50% and expressed as U/mg protein.

Study of expression pattern of antioxidant genes as biomarkers

Cell has a battery of antioxidant proteins that get expressed under stress condition.The expression pattern of antioxidant genes such as GST, CAT, SOD and GPX will be studied at both mRNA levels and protein levels by means of Quantitative Real Time PCR and Western blotting technique.

Real Time Reverse Transcription-Polymerase Chain Reaction (Q-PCR)

Total RNA will be isolated from frozen tissues using the TRIzol reagent (Sigma-Aldrich, MO, USA) according to the manufacturer's protocol. The concentration of total RNA will be quantified by absorbance at 260 nm using a BECKMAN COULTER Spectrophotometer (DU 730). The purity of total RNA will be assessed by determining the A260/A280 ratio. 5 µg of total RNA will be reverse-transcribed into cDNA using the Revert Aid reverse transcriptase (Fermentas) following manufacturers protocol. Quantitative real-time PCR will be performed on

a BioRad CFX Manager qPCR system. The 20 µL reaction mixture contained 10 µL of 2× iQ SYBR Green Supermix (Bio-Rad, USA), forward and reverse primers (0.1 µM each), nuclease-free water, and 1 µL of cDNA template. As a house-keeping gene, β-actin transcript will be used. The mRNA level will be expressed as its ratio to β-actin mRNA. PCR will be performed in triplicate with appropriate primer sequences. The gene-specific PCR primer sequences will be either designed or taken from published report (Liedtke *et al.* 2008). The amplification protocol will be as follows: 95 °C for 10s followed by 40 cycles at 94 °C for 5s, 59 °C for 15s, and 72 °C for 10s. The relative expression ratio (R) was calculated based on the following equation: $R = 2^{-\Delta\Delta Ct}$, where C_t represents the cycle in which the fluorescence signal is significantly different from background and $\Delta\Delta C_t$ is $(C_{t,target}-C_{t,actin})$ treatment − $(C_{t,target}-C_{t,actin})$ control. R is a ratio between the expressions in the treated sample versus the expression in the control sample in comparison to the β-actin gene.

Western Blot Analyses

Protein (60 μg) from the lysates of control and treated cells will be resolved on 10% SDS-PAGE at a constant voltage (60V) for 2.5h and then transferred onto a poly vinylidene fluoride (PVDF) membrane using blot apparatus (Bio-Rad, USA). The membranes will be first incubated with primary antibodies at a dilution of 1:1000 overnight at 4^0C, followed by 2h incubation with corresponding ALP-linked goat anti-rabbit (for Nrf2 and Keap1) or anti-mouse IgG secondary antibodies (for β-actin) at 1:5000 (Sigma) dilutions with continuous rocking. The immunoreactive bands will be detected using 5-bromo-4chloro-3-indolylphosphate/nitrobluetetrazolium (BCIP/NBT). Densitometric quantification will be done using ImageJ (NIH) software.

Cell viability assays in cells from Zebrafish liver:

Single cell suspension of liver will be prepared from liver of both treated and non treatedzebrafish. The cell suspension will be subjected to following cell viability assays .

Trypan blue assay:

The cell suspension prepared from liver tissue will be simply mixed with trypan blue dye and visually examined with the help of haemocytometer. Living cells possess intact cell membranes that extrude trypan blue dye, where as dead cell do not.

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium reduction assay:

To address this issue cell suspension is prepared from liver tissue, which is then subjected to treatment with MTT. Viable cells with active metabolism convert MTT into a purple colored formazan product. The quantity of formazan (presumably directly proportional to the

number of viable cells) is measured at 595 nm using a plate reading spectrophotometer. Percentage of viable cells will be calculated taking the viability of the untreated cells as 100%.

In vitro toxicity studies using Human Peripheral Blood Mononuclear Cells (HPBMCs):

Fresh blood will be drawn from the healthy human donor. HPBMCs will be isolated. For this purpose, blood will be diluted with equal volume of PBS and layered over Histopaque followed by centrifugation. The buffy coat will be aspirated into PBS for centrifugation. Cells will be washed thrice repeating this step. The rationale for using peripheral blood samples from healthy human volunteers is to reflect toxicity analysis in humans.

Determination of lethal concentration 50 (LC50) by Trypan blue dye exclusion assay:

The *in vitro* cytotoxic effects of arsenic and fluoride will be estimated by the Trypan blue dye exclusion. Cells will be counted using a hemocytometer, and the number of viable (opaque) and nonviable (stained blue) cells will be scored by direct observation under a bright-field microscope. The results will be subjected to probit analysis.

In the cytotoxicity assay, various aliquots of arsenic and fluoride will be added to the blood samples and incubated for 2 h. The viability of the lymphocytes will be recorded. The concentrations and percent viability data will be subjected to probit analysis using the probit model program and LC50 of the test compound will be calculated. $1/10^{th}$concentration of determined LC50 of arsenic and fluoride can be used as treatment concentration for further toxicity assays.

Cell proliferation study by MTT assay:

Isolated HPBMCs will be stimulated by Phytohaemagglutinin M (PHA) and cultured in RPMI-1640 media with 10% FBS along with antibiotics. After24 h, different concentration of individual and combined exposure of arsenic and fluoridewill be added in triplicate and cultured for another 48 h. Then MTT will be added and cell viability will be checked in a spectrophotometer. Percentage of viable cells will be calculated taking the viability of the untreated cells as 100%.The assay is based on the reduction of the MTT, a yellow tetrazole, to purple formazan by cellular mitochondrial dehydrogenases in living cells. Expansion in the number of viable cells resulted in an increase in the activity of the mitochondrial dehydrogenases, which leads to the increase in the amount of formazan dye formed. The formazan dye produced by viable cells can be quantified spectrophotometrically.

Apoptosis detection via Hoechst staining:

Nuclear fragmentation along with DNA degradation is the preliminary detecting sign of apoptosis induction. In this study cells will be fixed in 4% paraformaldehyde after treatment with different concentration of individual and combined exposure of arsenic and fluoride. Cells will be placed on glass slides and dried in air, stained with Hoechst and washed with PBS. Stained cells will be examined under a fluorescent microscope and analyzed for nuclear breakage.

Cell cycle analysis by FACS:

For flow cytometry, cells from both treated and untreated control will be mixed with phosphate buffered saline (PBS) and centrifuged. Then it will be resuspended in RBC lysis buffer and will be kept in ice. Following another centrifugation, cells will be washed, fixed, and permeated with ice-cold methanol. Then RNase and propidium iodide per sample will be added and incubated in the dark. The samples will be analyzed by a flow cytometer and data acquisition and processing will be performed with particular software.

Cellular toxicity detection by different redox enzyme activity assays:

Both treated and untreated control HPBMCs will be assayed for various enzymatic activities for detecting cellular oxidative stress level.

Chromosomal aberration studies to evaluate genotoxic potentials:

HPBMCs will be cultured and metaphase plates will be prepared after treatment with different concentration of individual and combined exposure of arsenic and fluoride to detect their genotoxic potentials.

Methodology: Fresh heparinized blood was used from three healthy male (20-25 years of age) not addicted to smoking or alcohol.Blood was drawn by trained medical staffs in the University hospital only. For each experimental group, triplicate cultures were maintained. Phytohemeagglutinin M (0.1mg/mL) was added to whole blood (0.5 mL) and then 5 mL of culture media (RPMI 1640) was added to it supplemented with 10□ fetal bovine serum along with penicillin (20µg/mL) and streptomycin(20U/mL).The culture tubes were kept at 37˚C .After 24h, aqueous solution of arsenic and fluoride (individual and combined) were added at different concentrations.BSO(5mM) and NAC(2mg/mL) were added 5h and 30 min prior to arsenic and fluoride treatment respectively.At 69 h of culture, colcemid(0.04µg/mL) was added and after three hours cells were harvested for metaphase chromosome preparation. The cultured blood cells will be harvested by centrifugation at 1500 rpm for 5 min then the cells will be suspended in 0.75M KCl (prewarmed at 37˚C,hypotonic solution).Cells will be treated in the hypotonic solution for 22 min. After that the cells will be fixed in acetic acid and methanol (1:3). Then the cell pelltes will be resuspended in 1mL of fixative, dropping onto a grease free chilled slide and flame dried. Slides will be prepared by flame drying method and stained in 5□ Giemsa for 5 min and mounted in a synthetic mountant and evaluated under light microscope.

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